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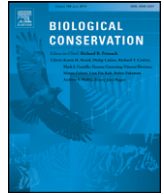
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The genetic consequences of long term habitat fragmentation on a self-incompatible clonal plant, *Linnaea borealis* L.

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ABSTRACT

Self-incompatible species with restricted seed and pollen flow are considered the most vulnerable to the deleterious genetic effects of habitat fragmentation. Immediate effects of fragmentation are expected to be loss of allelic diversity and differentiation of fragments by genetic drift. Later, loss of S allele diversity may lead to restricted mate availability, increased relatedness of genotypes within patches, accelerated loss of genetic diversity and eventual loss of capacity for seed production. We studied the self-incompatible clonal shrub *Linnaea borealis* within the Cairngorms National Park, Scotland, whose pinewood habitat has been fragmented for an extensive period, possibly millennia. Exhaustive sampling revealed 123 patches (median length 15 m), 91% of which were further than the maximum pollen flow distance from their nearest neighbours (30 m). Using ten microsatellite markers, only 21% of the patches produced more than one multilocus genotype. Individual genotypes extended from 1 to 74 m. Bayesian clustering of the 179 multilocus genotypes revealed six clusters. One cluster occupied a geographically distinct area where seed production still occurs and showed significant genetic differentiation from ($F_{st} = 0.164$, $P < 0.01$) and significantly lower allelic richness ($A_R = 4.0$ vs $A_R = 7.0$ $P < 0.01$) than the remainder of the sample set. Spatial genetic structure in the total sample set indicated significant relatedness of clones within the first 1.5 km. Overall, *L. borealis* in Scotland seems to be experiencing extreme genetic effects of chronic population fragmentation with only 16% of patches having the capacity for seed production. Genetic rescue is being undertaken by transplanting unrelated clones from >1.5 km distance into extant monoclonal patches.

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1. Introduction

Habitat fragmentation is widely cited as a major cause of plant species declines through its effects on both the genetics and demography of populations (Barrett and Kohn, 1991). In terms of genetics, habitat fragmentation has the potential to restrict gene flow via both pollen and seed, creating a series of sub-populations with small effective population size within which localised inbreeding and loss of genetic variation occurs (Ellstrand and Elam, 1993). Reduction in fitness due to inbreeding depression and loss of evolutionary potential due to genetic drift may then combine with demographic stochasticity to generate local extinction vortices within each of the sub-populations cut off by fragmentation (Frankham, 2005).

The extent to which habitat fragmentation produces the deleterious genetic effects outlined above depends crucially on details of the breeding system and reproductive biology of the taxon concerned (Wagenius et al., 2007; Thrall et al., 2014). Deleterious genetic effects of habitat fragmentation are expected to be minimal in the many species that possess the potential for long distance pollen and seed flow capable of maintaining genetic communication among remnant sub-populations. This is evidenced by the numerous studies on forest trees in which population fragmentation may actually increase seed and pollen gene flow across the landscape (Kramer et al., 2008; Bacles and Jump, 2010; Lowe et al., 2015). Genetic effects of habitat fragmentation will also be less severe in predominantly inbreeding species which carry little genetic load due to segregation of deleterious recessive mutations and are capable of producing seed via self-fertilisation with little or no fitness cost (Wagenius et al., 2007).

In contrast, the genetic effects of fragmentation are likely to be most severe in plant species with restricted pollen and seed flow that possess a self-incompatible mating system (Young et al., 2012; Thrall et al., 2014). This combination of traits is commonly found in herbaceous

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species with animal dispersal of pollen, primary dispersal of seed by gravity and the potential for extensive clonal spread. In such species, self-incompatibility is likely to have evolved because of the fitness advantage conferred by prevention of geitonogamous self-fertilisation (Vallejo-Marín et al., 2010). A predictable series of changes in genetic structure are anticipated to occur if population fragmentation is imposed on a large continuously distributed population of a self-incompatible clonal species with restricted seed and pollen flow.

Before fragmentation, the population comprises a large number of interspersed genetically diverse clones with high S allele diversity. Although pollen flow is restricted, it will be sufficient to allow compatible matings between clones, seed production and associated gene flow within the population by seed, and recruitment of unrelated genotypes within any part of the population. The combined gene flow via pollen and seed will generate a large genetic neighbourhood size (Crawford, 1984; Epperson, 2007). While there will be some isolation by distance across the population there will be little opportunity for genetic drift to lead to marked genetic differentiation or loss of genetic diversity within particular geographic localities.

Fragmentation of the habitat will produce patches containing a relatively small number of genotypes of the self-incompatible clonal species. Patches may be sufficiently far apart that pollen and seed flow between them is not possible. Within the patches, pollination will take place between existing clones, and seed will disperse within the patch. However over subsequent generations, due to the small effective population size of these patches, genetic variation will be lost from them. Consequently, genotypes within the patches will become increasingly closely related, and genetic differentiation among the patches will occur through drift. In addition, the loss of self-incompatibility alleles by drift will increasingly restrict the availability of compatible clones, reduce seed set, increase the variance in male and female reproductive success and further decrease the effective size of the populations (Young et al., 2000; Wagenius et al., 2007). With loss of compatible mates, seed production, seed flow and sexual recruitment into the population will eventually cease. Thereafter, random or selective loss of clones may eventually result in patches becoming occupied by a single clone. In the absence of intervention, genotypes within these patches will be unable to reproduce sexually, and individual patches will be incapable of adaptive evolution in response to environmental change.

These considerations provide us with a framework for using information on the genetic structure of populations to assess the severity of effects which have resulted from habitat fragmentation in a self-incompatible clonal species with restricted gene flow. If fragmentation effects are at an early stage we expect to see multi-clonal patches showing successful seed production despite their genetic isolation. However as a result of genetic drift these isolated patches will show genetic differentiation and reduced genetic diversity compared with the population as a whole. Additionally, genotypes will show greater relatedness to members of their local patch than to individuals located further away. This will be detectable through measurement of spatial genetic structuring. However if fragmentation has persisted for a considerable period, the prediction is that patches will become occupied by one or a few genotypes which no longer produce seed, and the extent of spread of these clonal genotypes via vegetative growth may be very large.

A classic example of a self-incompatible clonal plant with restricted gene flow that is considered to be suffering from the deleterious effects of habitat fragmentation is twinflower, *Linnaea borealis* L. *L. borealis* is an insect pollinated, animal dispersed, clonal understorey shrub with a circumpolar distribution (Wroblewska, 2013). In Scotland, although there are some records of remnant patches of *L. borealis* surviving on previously wooded heathland sites (Welch, 2003), it occurs predominantly within native Scots pine (*Pinus sylvestris* L.) woodlands (Lusby and Wright, 1996). Here the overstorey of tree cover provides the environmental conditions for long term persistence. Over the last 4000 years, and increasingly over the past four centuries, the once extensive and continuous pinewood habitat in Scotland suitable for *L. borealis* has

been reduced to <1% of its former area. Moreover, habitat suitable for *L. borealis* is now highly fragmented with pinewood remnants typically being small and separated from each other by considerable distances (Steven and Carlisle, 1959; Anderson, 1967; Kinloch et al., 1986).

Previous work on the reproductive biology of *L. borealis* in Scotland and China has shown that the species is highly self-incompatible and that effective pollen dispersal occurs over no >30 m. (Wilcock and Jennings, 1999; Scobie and Wilcock, 2009; Zhang et al., 2014). There are already widespread reports of failure of seed production within Scottish *L. borealis* populations, and preliminary estimates of clonal diversity, based on morphological traits and isozyme markers in a limited number of sites, detected no more than one genotype in 37% of patches (Wilcock and Jennings, 1999; Kohn and Ennos, 2000; Scobie and Wilcock, 2009). Experimental evidence from controlled pollen transfer has unequivocally demonstrated that failure of seed set in studied patches is due to lack of compatible genotypes within effective pollination distance, rather than lack of pollinator service (Scobie and Wilcock, 2009). Thus both the reproductive biology of *L. borealis* and ecological history suggest that it is subject to, and has potentially been suffering from, the deleterious genetic effects of habitat fragmentation for a very long period of time.

The aim of the present study is to use a combination of landscape scale mapping of populations and population genetic analysis to determine the effect that chronic fragmentation of habitat has had on *L. borealis* in Scotland. We first document the physical fragmentation of the *L. borealis* population by establishing the size and distribution of all remaining patches of *L. borealis* within its core habitat in the Cairngorms National Park. We then apply a recently developed set of micro-satellite markers to multiple samples of *L. borealis* from each of these patches to estimate the clonal diversity within patches, and to assess the clonal patch size. Having identified clonal genotypes, the genetic data are then used to establish the distribution of distances among clones, and to determine whether genotypes in spatial proximity show elevated levels of relatedness. Finally, we ask whether there is any evidence of geographically discrete groups that show genetic differentiation from and reduced genetic diversity compared to the rest of the sample set, a situation expected in the early stages of fragmentation. The results are combined to build up a complete picture of the effects of population fragmentation on *L. borealis* within its core distribution in Scotland. This information is used to provide management guidelines for alleviating the deleterious effects of past fragmentation on the extant Scottish *L. borealis*.

2. Materials and methods

2.1. Study species

L. borealis is a small, creeping, evergreen, perennial shrub which has a circumpolar distribution in the forests of North America, Europe and Asia. Although common in many parts of its range, in Britain it is restricted to Scotland where it is both local and rare (Lusby and Wright, 1996). The species is diploid ($2n = 32$) and self-incompatible. The small bell shaped flowers are pollinated predominantly by flies and each may produce a maximum of two seeds. The fruits possess sticky glandular hairs which aid their dispersal by animals.

2.2. Sampling

All 123 patches of *L. borealis* known to exist in the Spey and Dee river catchments within the Cairngorms National Park in Scotland, UK ($57^{\circ}5'N$, $3^{\circ}4'W$, Total area 4528 km²) and documented in the Cairngorms Rare Plant Project (http://www.cairngormsrareplants.org.uk/index.php/the_project) were sampled. To maximise the chance of detecting different clones efficiently, while simultaneously measuring the extent of clones, the number and location of samples taken from a patch varied depending on patch size; two samples were taken from opposite ends of

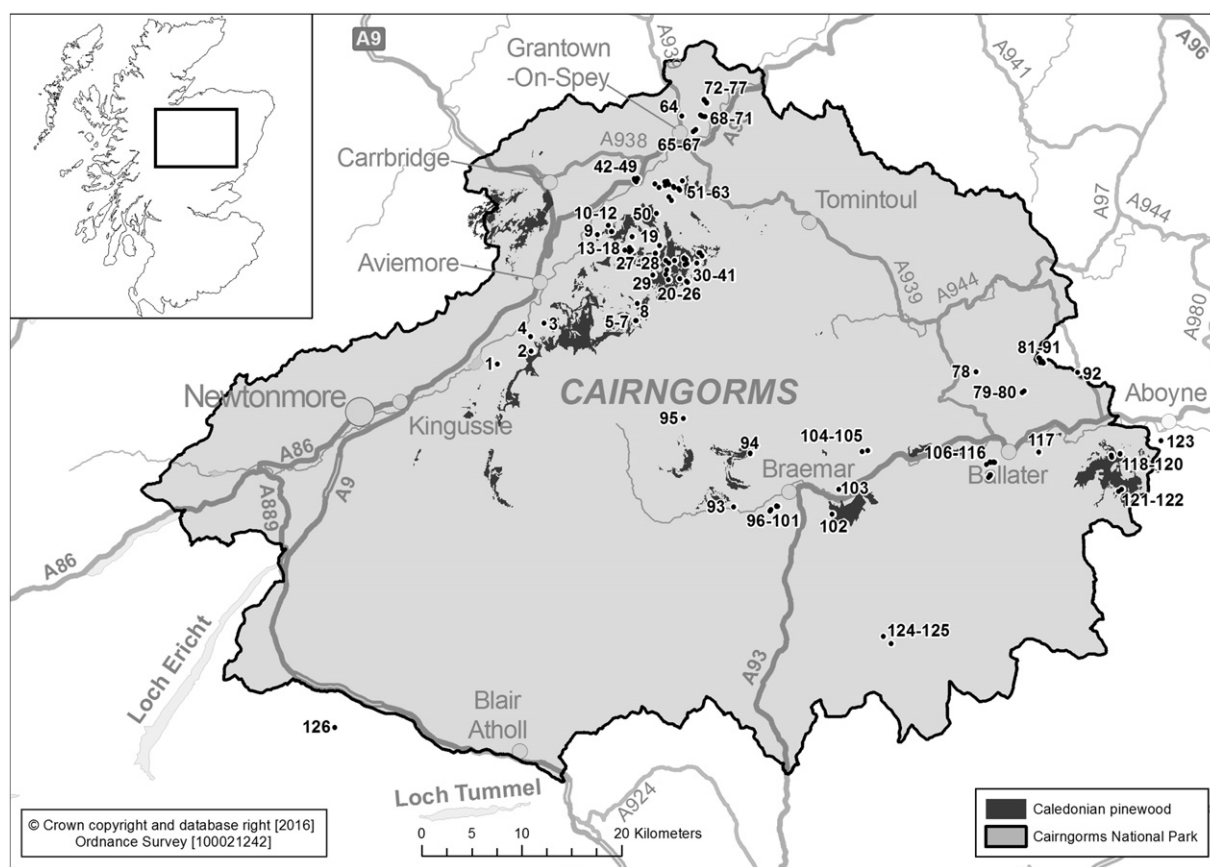


Fig. 1. Map of the Cairngorms National Park showing the locations of the sampled patches of *L. borealis*. The locations of the three additional patches which were entered into the cluster analysis are also shown. Shaded areas indicate distribution of Caledonian pinewood remnants. Inset map shows the geographic location of the sampling area in Scotland.

patches which measured $<10\text{ m} \times 10\text{ m}$ and five samples taken from the four opposite corners and the centre of patches that exceeded this size. In some of the larger patches additional samples were also taken. Using this rationale, a total of 410 samples, each comprising 10 cm of stolon plus leaves were collected and their GPS locations recorded to an accuracy of one meter. Samples were stored in silica gel prior to DNA extraction. Details of the sample locations are provided in Fig. 1 and Table A1 (Supplementary material).

2.3. Microsatellite marker analysis and clonal assignment

DNA was extracted from a single leaf using a Qiagen DNeasy kit (Qiagen.com) and samples were genotyped at ten microsatellite loci (A5, A102, A112, B119, C105, A112, D7, D110, D110a, D118) as described by A'Hara et al. (2012). The identity and number of multilocus genotypes present in the set of samples was determined using GenClone v2.0 (Arnaud-Haond et al., 2007). The probability of genetic identity of two individuals following sexual reproduction was calculated using GenAlEx 6.5 (Peakall and Smouse, 2012). This clone corrected data set was then used to estimate genetic diversity parameters for the total population in FSTAT v. 2.9.3.2. (Goudet, 2002).

2.4. Patch size and degree of population fragmentation

Patch size was measured as the length of the longest axis of a patch [m]. The length of the longest axis of a patch was used as a patch size estimator rather than the number of flowers or flowering individuals due to the morphology of *L. borealis*. The plant does not occur as discrete flowering individuals, but as a more or less continuous mat of stolons spreading across the ground and among under storey shrubs with

occasional flowers located along those stolons. In these circumstances the maximum extent of ground covered by the plant, here measured as the longest axis of the patch gives the best idea of the size of the patch of *L. borealis*. Patch size was determined from the sample GPS coordinates from each patch. To document the degree of habitat fragmentation, the distribution of distances from each patch to the nearest population of *L. borealis* was measured.

2.5. Clone size and nearest neighbour distance

Minimum clone size [m] was determined from the maximum distance between two samples possessing the same multilocus genotype. Nearest neighbour distance for a multilocus genotype was measured as the distance [m] to the nearest sample of a distinct multilocus genotype.

2.6. Spatial genetic structure in the total population

To explore genetic structure at a landscape scale the dataset was hierarchically divided, firstly into samples from the two main river catchments, the Spey and the Dee and secondly, into 15 geographic subsets within these. The data in this format were then subjected to Analysis of Molecular Variation (AMOVA) analysis using the Arlequin v. 3.5.1.2 software (Excoffier et al., 2005) to determine the geographic scale at which genetic variation was partitioned.

The program SPAGeDi v1.4 (Hardy and Vekemans, 2002) was used to determine whether there was any evidence within the total population for greater relatedness of clones found in spatial proximity. Average pairwise Loiselle kinship coefficients (Loiselle et al., 1995) were calculated using the clone corrected data set across 40 distance classes

1.5 km wide from 0 to 60 km. In addition, 1000 permutations were performed to obtain a 95% confidence interval (CI) for the coefficient under the null hypothesis of no spatial structuring.

2.7. Evidence for sub-population genetic divergence

To establish whether there was any evidence for localised genetic divergence of sub-populations the clustering algorithm in the adegenet package available as an R package (Jombart, 2008) was used to find the optimum number of genetic clusters in the clone corrected data set. Three additional patches (Patch numbers 124, 125 and 126) that were outwith the main Spey and Dee river catchments were included in this analysis, making a total of 126 patches. The geographical location of individuals in each of these clusters was then inspected to determine whether any of these clusters were located in patches occupying a limited geographic area. For any cluster showing a localised spatial distribution we used FSTAT Version 2.9.3.2 (Goudet, 2002) to calculate the Weir and Cockerham (1984) estimate of Wright's F_{st} to measure the extent of genetic divergence of that cluster from the remainder of the population. We used a general linear model and an a posteriori test in statistical software package MINITAB Version 16 to compare the allelic richness of that cluster with that of all other clusters in the population.

3. Results

3.1. Microsatellite marker diversity and clonal assignment

Of the 410 ramets sampled, 350 were successfully genotyped at all ten microsatellite loci, 52 were genotyped at nine loci, and the remaining eight at eight loci. All microsatellite loci were polymorphic with number of alleles per locus ranging from 9 to 22 (mean = 12.6). Gene diversity in the sample set of 123 patches was, $H_T = 0.716$. Even with only eight loci scored, the probability of genetic identity of two individuals following sexual reproduction was as low as 1.3×10^{-8} , indicating that samples with identical multilocus genotypes could be unequivocally assigned as ramets of the same clone. Analysis of the number of unique multilocus genotypes in the sample indicated the presence of 179 genets in the Cairngorms National Park population. In small patches where only two samples were taken, the proportion of patches in which multiple genotypes were found was 0.18. In large patches where five samples were taken, the proportion of patches in which multiple genotypes were found was 0.20. The similarity of these values, given that the opportunity for finding multilocus genotypes in larger patches is higher than in smaller patches, suggests that taking only two samples in small patches did not lead to serious underestimation of the frequency of multi genotype patches. Subsequent analysis of genetic variation and genetic structure (Loiselle kinship coefficient) within *L. borealis* was based on this clone corrected data set.

3.2. Patch size and degree of population fragmentation

There was a wide distribution of patch sizes of *L. borealis* within the Cairngorms National Park, with the longest axes of patches ranging in size from 1 m to 105 m. On average, however, *L. borealis* occurred in patches of small size (Median = 15 m, Fig. 2a). The distribution of distances from a patch to its nearest neighbour was also variable (Fig. 2b), but most patches (91%) were separated from their nearest neighbour by a distance greater than that over which pollen has been shown to be effective (30 m). There was no evidence to indicate that small patches were further away than large patches from their nearest neighbouring patch although single clone patches were on average 264 m away from their nearest neighbouring patch compared to an average of 109 m for multiclonal patches.

3.3. Clone size, clonal diversity of patches and nearest partner distances

Fig. 2c shows the distribution of minimum length of the longest axis of clones inferred from the mapped ramets. Recorded length of clone varies from 1 m to 74 m, with a median of 10 m. Individual clones were confined to a single patch except in two cases where they spanned two adjacent patches (pooled for analysis due to presence of a single genotype) at Carn a Chnuic 1 & 2 (patch 32, 54 m apart) and Morven East 4 & 5 (Patch 84, 39 m apart).

The distribution of number of clones/patch detected in the total sample of 123 patches is shown in Fig. 3a. The proportion of patches in which only one genotype was detected was very high, 79%, and did not differ significantly between small ($<10 \text{ m} \times 10 \text{ m}$, 2 ramets genotyped) and large ($>10 \text{ m} \times 10 \text{ m}$, 5 ramets genotyped) patches ($\chi^2_{(1)} = 0.10$, $P > 0.05$). The highest numbers of clones/patch were detected at Old Grantown Wood (patch 64, 5 clones) and Anagach Wood 2 (patch 66, 5 clones) each, Birkhall 2 (patch 112, 8 clones) and Birkhall 3 (patch 116, 6 clones).

Fig. 3b shows the distribution of distances between detected clones and their nearest potential mating partner. Only 6% of clones have neighbouring genotypes within 6 m, the maximum distance within which pollen flow is fully effective for seed set. 39% of clones have neighbours within 30 m, the maximum distance at which pollen flow produces any measurable seed set (Scobie and Wilcock, 2009).

3.4. Spatial genetic structure of population

At a landscape scale, the proportion of total genetic variation accounted for by separation into the two river catchments was only 0.5% while variation among the 15 geographic localities within catchments accounted for 5.0%.

The variation in relatedness (measured by the Loiselle kinship coefficient) with distance between genets for the whole Cairngorms population is shown in Fig. 3c. In the shortest distance class genotypes are strongly related with a kinship coefficient of over 0.125. This drops off very sharply to become non-significant when the distance between genotypes is $>1.5 \text{ km}$.

3.5. Sub-population genetic divergence and genetic diversity

Cluster analysis in Adegenet provided evidence for six genetic clusters in the total data set. A scatterplot of the Discriminant Analysis of Principal Components (Jombart, 2008) indicates that one of these six clusters, cluster 5 containing 15 genotypes, is distinct from all other clusters on the first discriminant analysis (Fig. 4 and Fig. A1 (Supplementary material)). All individuals in cluster 5 fall within four adjacent patches, Birkhall 1 (1/1), Birkhall 2 (6/8), Birkhall 3 (6/6) and Birkhall 5 (2/2) (Table A1, Patches 111, 112, 116 and 114). The genotypes within cluster 5 possess no unique alleles. However, cluster 5 shows genetic differentiation from the remaining genotypes in the sample set ($F_{st} = 0.164$, $P < 0.01$) with mean pairwise F_{st} for cluster 5 ($F_{st} = 0.211$) being higher ($P < 0.05$) than that for all other clusters (mean pairwise $F_{st} = 0.122$) (Table 1). In addition allelic richness of genotypes in cluster 5 ($A_R = 4.0$) is significantly lower ($P = 0.001$) than in the remainder of the population as a whole ($A_R = 6.987$) and below that of all other individual clusters (Mean $A_R = 6.4$) ($P < 0.05$ a posteriori test, Table 1).

4. Discussion

L. borealis within the Cairngorms National Park shows all the ecological and genetic signatures of a species suffering from the most extreme and deleterious effects of habitat fragmentation. The population is currently subdivided, by loss of pinewood habitat, into a collection of spatially isolated patches whose mean size is very small (median length 15 m). For the vast majority of these patches (91%) the distance to the nearest patch is greater (often much greater) than 30 m, the maximum

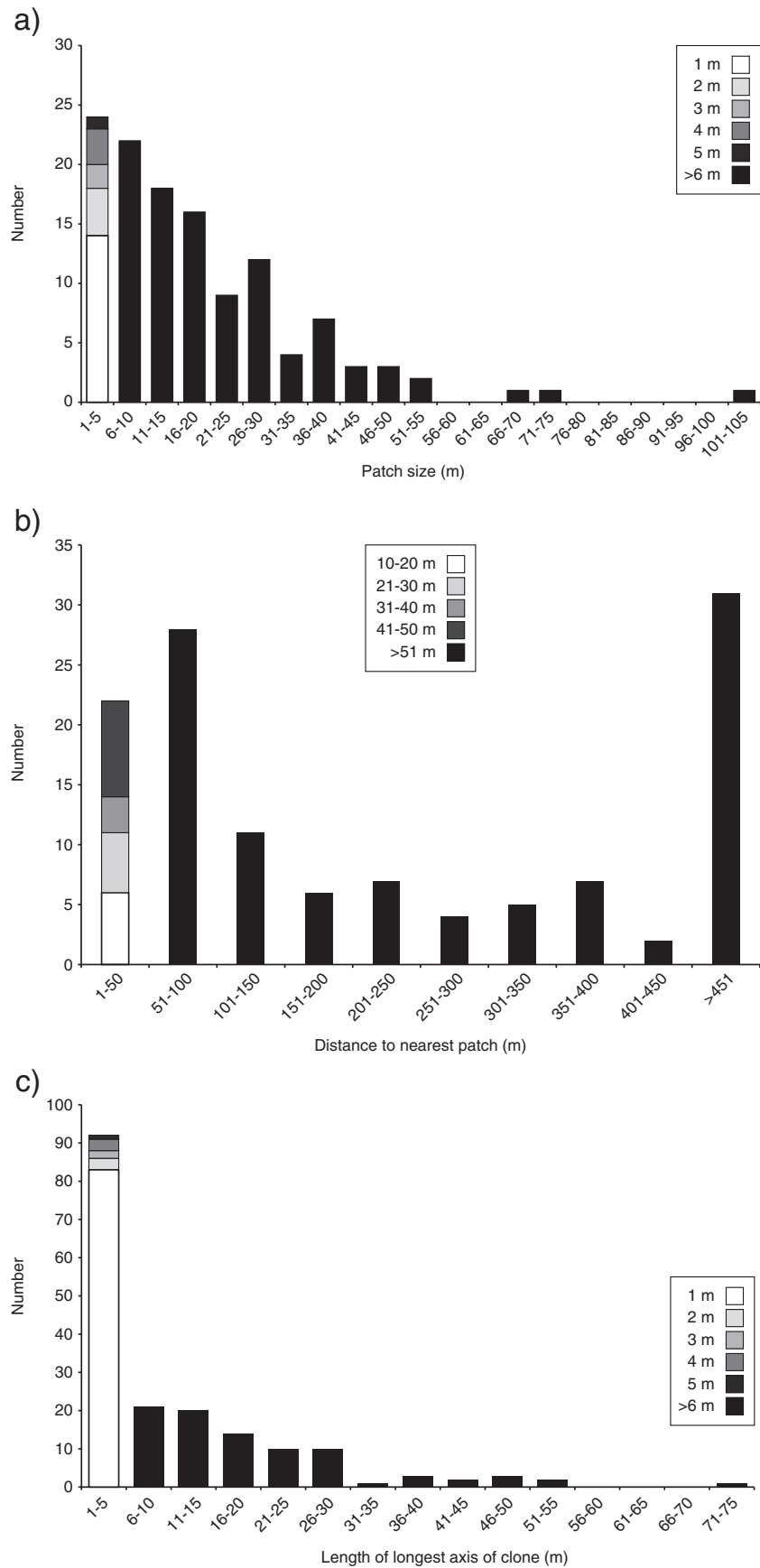


Fig. 2. Distribution of a. patch sizes (length of longest axis [m]) for the patches of *L. borealis* sampled in the Cairngorms National Park (the first size class is broken down into one meter units); b. nearest patch neighbour distances [m] (the shortest distance class is subdivided into units of 10 m, there were no neighbours within a distance of < 10 m), c. lengths of the longest axis for the 179 *L. borealis* sampled clones (the distribution in the first size class is divided into one meter units).

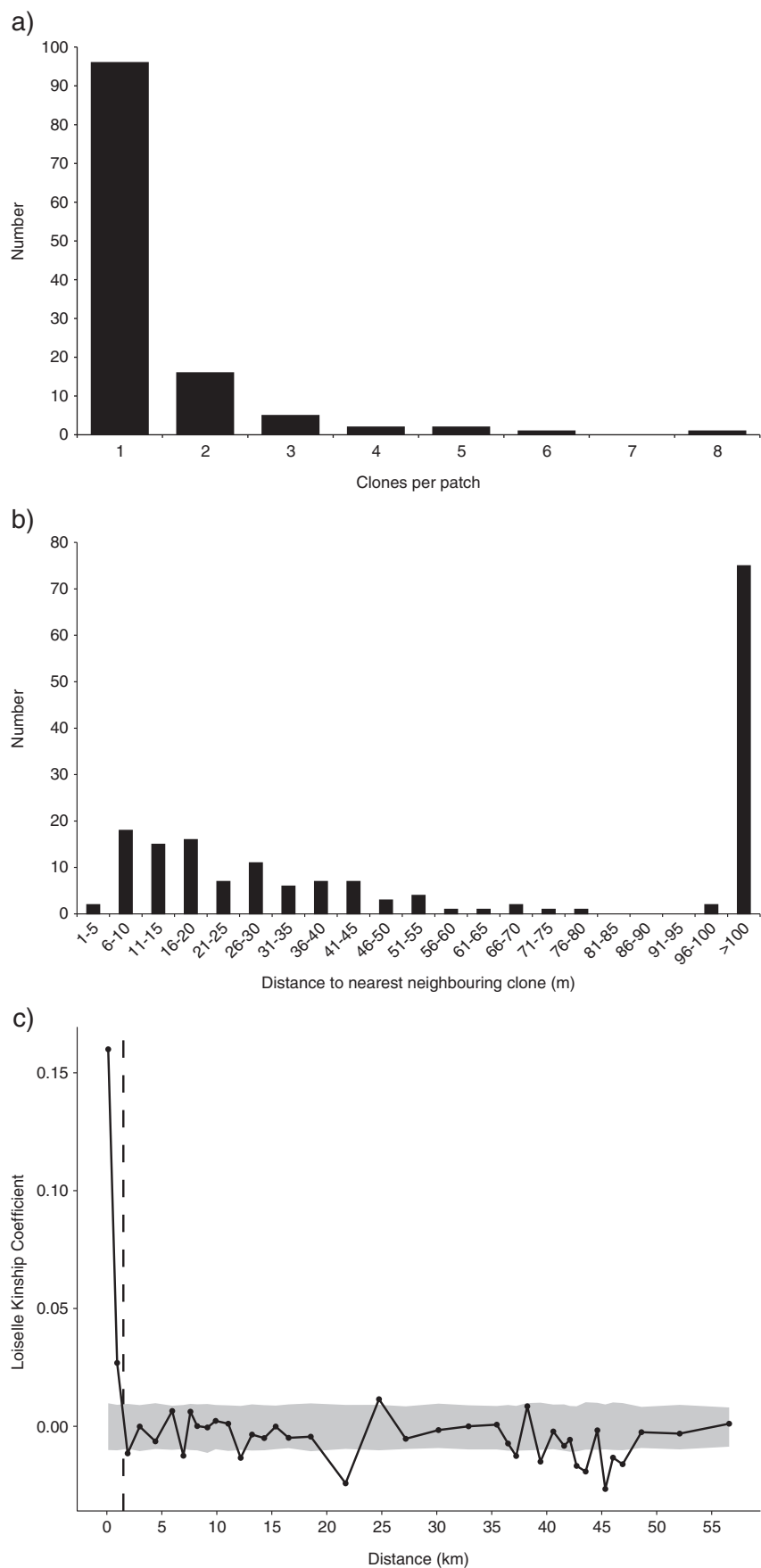


Fig. 3. Distribution of a. number of clones detected within each of the patches of *L. borealis* in the Cairngorms National Park, b. nearest clone neighbour distances for the 179 *L. borealis* sampled clones, c. Variation in kinship coefficient (Loiselle et al., 1995) between pairs of clones of *L. borealis* present within different distance classes. Shaded area represents 95% confidence interval under the hypothesis of no relatedness.

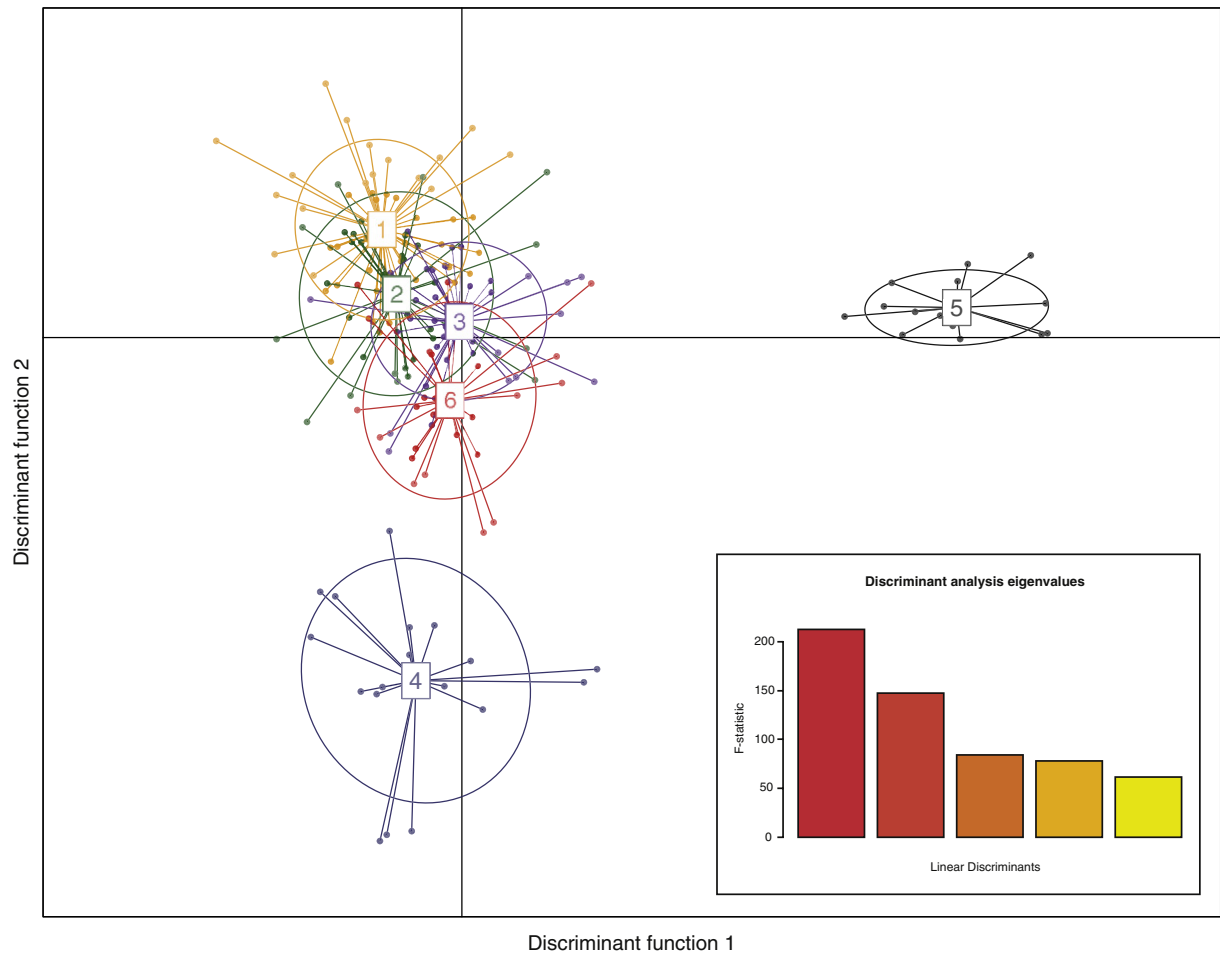


Fig. 4. Scatterplot for Discriminant Analysis of Principal Components (DAPC) for 179 multilocus genotypes of *L. borealis* from the Cairngorms National Park clustered into 6 clusters (Jombart, 2008). The x and y axes represent the discriminant functions one and two respectively. The inset bar chart provides the relative eigenvalues with the dark and light pink columns showing the first two discriminant functions that were used in the analysis.

distance over which any significant pollen flow is likely to occur (Scobie and Wilcock, 2009). Furthermore, in 78% of the patches analysed in this study only one self-incompatible genotype has been detected. Under the best case scenario where pollen dispersal is effective over as much as 30 m, and all distinct multilocus genotypes are compatible with one another, the total proportion of patches capable of producing seed is only 16%. We can conclude that the vast majority of the current population of *L. borealis* in its core area in Scotland is sustained through vegetative reproduction in isolated patches containing one or a few clones, within which there are no immediate prospects for future seed production.

Analysis of the genetic structure of the population provides insight into the processes by which the current situation has arisen. Cluster analysis of genetic data from the complete population reveals a set of

adjacent multilocus patches at Birkhall, on the Balmoral Estate, that are both genetically differentiated from the remainder of the population and have significantly reduced allelic diversity. Previous studies have indicated that these patches contain sufficient clonal and S allele diversity to sustain seed production (Scobie and Wilcock, 2009). Indeed, Scobie and Wilcock (2009) recorded values of 33.8 and 38.6% natural fruit set in two patches at Balmoral considered, on the basis of differences in flower colour, to be multiclinal. These values were much higher than those of 1.8–8.1% for single clone patches at Balmoral and at two sites elsewhere in the Cairngorm National Park. Our genotyping results confirm that patches at Birkhall contain particularly large numbers of clones and it appears that the patches in the Birkhall area are continuing to pass through sexual generations. However, because their effective population sizes are small they are both differentiating and losing allelic diversity every generation as a consequence of genetic drift. If this process of localised differentiation has taken place more widely in the past, it is anticipated that genotypes in spatial proximity throughout the population should show increased relatedness. Spatial genetic analysis of the total population shows a strong and significant increase in relatedness of individuals within but not beyond a distance class of 1.5 km., providing support for this prediction.

In a self-incompatible species such as *L. borealis*, one of the effects of genetic drift within patches with low effective population size will be the loss of S alleles (Pickup and Young, 2008; Young and Pickup, 2010). In conjunction with limited pollen flow this will give rise to a genetically based Allee effect where seed production becomes limited by

Table 1

Mean pairwise genetic differentiation (F_{ST}) between and allelic richness (A_R) within each of six genotype clusters of *L. borealis* in the Cairngorms National Park. Values of A_R with different letters are significantly different ($P < 0.05$).

| CLUSTER | F_{ST} | A_R |
|---------|----------|-------|
| 1 | 0.130 | 6.6a |
| 2 | 0.121 | 6.3a |
| 3 | 0.101 | 7.0a |
| 4 | 0.152 | 5.8a |
| 5 | 0.211 | 4.0b |
| 6 | 0.108 | 6.4a |

paucity of compatible genotypes in the patch. This will become increasingly important as the patch ages to the point where no S allele diversity exists in the oldest patches (Wagenius et al., 2007). If no change in the breeding system to self-compatibility occurs, genotypes within patches can only be sustained by vegetative reproduction. This appears to be the case for the vast majority of the remaining patches of *L. borealis* in Scotland where compatible mating partners are absent within patches. This extreme scenario is consistent with the length of time, possibly as long as four thousand years, over which fragmentation of the pinewood habitat appears to have been ongoing in Scotland (Anderson, 1967). A comparable situation of loss of S alleles within patches is found in the lakeside daisy *Hymenoxys acaulis* var. *glabra* which has been confined to restricted calcareous habitat for the past 4000 years. Here all individuals from remnant patches within Illinois are of the same incompatibility type (DeMauro, 1993).

This situation in *L. borealis* and *H. acaulis* contrasts with that found in once widespread incompatible species whose distribution has been fragmented in much more recent times. Thus, in *Rutidosia leptorrhynchoidea* whose reduction in population size began in 1874, loss of S alleles has occurred in small populations. Here, there is evidence of some mating partner restriction and associated higher variance in male and female fitness. However, despite some loss of allelic richness small populations still contain a high number of compatible genotypes (Young et al., 1999, 2000; Pickup and Young, 2008; Young and Pickup, 2010). Similarly, in small populations of self-incompatible *Echinacea angustifolia* created in the 19th century by prairie fragmentation, loss of S alleles causes genetically based Allee effects, but compatible mates still occur within remnants (Wagenius et al., 2007).

In a self-incompatible clonal species subject to fragmentation, the point at which a switch occurs from mixed sexual and vegetative reproduction to exclusive vegetative reproduction will depend on initial S allele diversity within a patch. If this is high, populations could pass through many sexual generations before the switch occurs. Over these sexual generations substantial differentiation and loss of diversity at neutral genetic markers could take place. If S allele diversity is initially low, the switch to vegetative reproduction could take place after a few generations, and levels of genetic diversity at neutral markers could be retained without substantial loss in the clonal population that survives.

In the population of *L. borealis* within Scotland it is notable that neutral genetic diversity within the total population is very high at microsatellite loci ($H_T = 0.7$) and high diversity was also found in a previous study using isozyme markers ($H_T = 0.48$) (Kohn and Ennos, 2000). Similarly, high values of total microsatellite marker variation ($H_T = 0.75$) have been found in the clonal, self-incompatible herb *Convallaria majalis* in deciduous forests that have been long fragmented within Belgium (Vandepitte et al., 2010). Here many 6 m × 6 m areas sampled within patches contained single clones. In both cases this suggests a rapid switch to reliance on asexual reproduction before substantial loss of neutral marker diversity has occurred. In contrast, Les et al. (1991) found very low diversity at isozyme markers within the total population and very high differentiation between sites occupied by the self-incompatible *Aster furcatus*. This suggests that fragmented populations of this species passed through many sexual generations under fragmentation before they became reliant on vegetative reproduction for population maintenance.

A remarkable feature of *L. borealis* is its enormous capacity for vegetative spread. This attribute is conferred by its extensive creeping shoot system, with single genetic individuals often composed of hundreds of ramets (Eriksson, 1988, 1992). In this study a single genet extending over a distance of 74 m was found. With such a high ability for spread there is a finite possibility, over protracted periods of time, for currently isolated genets to meet once again as a consequence of vegetative growth and for sexual reproduction to resume. However, with 91% of patches being >30 m apart, this is likely to be a very rare occurrence. In these circumstances the only way to restore sexual reproduction, and evolutionary potential, to existing patches of *L. borealis* is artificially

to transplant compatible genotypes from elsewhere within pollination range (Wilcock and Jennings, 1999; Willi and Fischer, 2005; Pickup and Young, 2008; Scobie and Wilcock, 2009; Young and Pickup, 2010).

In a genetic rescue programme of this type, the priorities must be to ensure that the transplanted genotypes contain novel S alleles, that they are as far as possible unrelated to the resident clones, and that they are adapted to the habitat into which they are transplanted. This study provides no information about patterns of local adaptation of *L. borealis*, and in such a situation, matching the environments of donor and transplant sites will be the best way to maximise the chance of transplants being adapted to their new site. However, this study does provide information about spatial patterns of relatedness of genotypes. Genotypes located within 1.5 km of each other show elevated levels of relatedness. There is therefore an increased probability that they will share S alleles and show reduced compatibility. In addition, the offspring of mating between them will be inbred, and as *L. borealis* is an outcrossing species likely to carry significant genetic load, offspring are likely to be of reduced fitness (Fischer et al., 2003). Thus if a programme of artificial transplantation is conducted to restore seed set within a patch, transplant genotypes should be sourced from patches >1.5 km away, but occupying as far as possible similar environmental conditions. Such a programme of artificial transplantation of *L. borealis* clones to restore seed set within patches, making use of these guidelines, is already underway in the Cairngorms National Park.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.biocon.2016.07.032>.

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References

- A'Hara, S.W., Scobie, A.R., Broome, A., Long, D., Cottrell, J.E., 2012. Development of microsatellite markers in twinflower (*Linnaea borealis* L.) a rare Scottish pinewood plant. *Mol. Ecol. Resour.* 12, 185–189.
- Anderson, M.L., 1967. *A History of Scottish Forestry*. 2. Thomas Nelson and Sons, London & Edinburgh.
- Arnaud-Haond, S., Duarte, C.M., Alberto, F., Serrão, E.A., 2007. Standardizing methods to address clonality in population studies. *Mol. Ecol.* 16, 5115–5139.
- Bacles, C.F.E., Jump, A.S., 2010. Taking a tree's perspective on forest fragmentation genetics. *Trends Plant Sci.* 16, 13–18.
- Barrett, S.C.H., Kohn, J.S., 1991. Genetic and evolutionary consequences of small population size in plants. In: Falk, D.A., Holsinger, K.E. (Eds.), *Genetics and Conservation of Rare Plants*. Oxford Univ. Press, New York, NY, pp. 3–30.
- Crawford, T.J., 1984. The estimation of neighbourhood parameters for plant populations. *Heredity* 52, 273–283.
- DeMauro, M.M., 1993. Relationship of breeding system to rarity in the Lakeside Daisy (*Hymenoxys acaulis* var. *glabra*). *Conserv. Biol.* 7, 542–550.
- Ellstrand, N.C., Elam, D.R., 1993. Population genetic consequences of small population size: implications for plant conservation. *Annu. Rev. Ecol. Syst.* 24, 217–242.
- Epperson, B.K., 2007. Plant dispersal, neighbourhood size and isolation by distance. *Mol. Ecol.* 16, 3854–3865.
- Eriksson, O., 1988. Variation in growth rate in shoot populations of the clonal dwarf shrub *Linnaea borealis*. *Holarct. Ecol.* 11, 259–266.
- Eriksson, O., 1992. Population structure and dynamics of the clonal dwarf-shrub *Linnaea borealis*. *J. Veg. Sci.* 3, 61–68.
- Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol. Bioinformatics Online* 1, 47–50.
- Fischer, M., Hock, M., Paschke, M., 2003. Low genetic variation reduces cross-compatibility and offspring fitness in populations of a narrow endemic plant with a self-incompatibility system. *Conserv. Genet.* 4, 325–336.
- Frankham, R., 2005. Genetics and extinction. *Biol. Conserv.* 126, 131–140.
- Goudet, J., 2002. FSTAT, A Program to Estimate and Test Gene Diversities and Fixation Indices. Available from <http://www2.unil.ch/popgen/softwares/fstat.htm>.
- Hardy, O.J., Vekemans, X., 2002. SPAGeDi: a versatile computer program to analyse spatial. *Mol. Ecol. Notes* 2, 618–620.
- Jombart, T., 2008. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24, 1403–1405.

- Kinloch, B.B., Westfall, R.D., Forrest, G.I., 1986. Caledonian Scots pine: origins and genetic structure. *New Phytol.* 104, 703–729.
- Kohn, D., Ennos, R., 2000. Action Plan Research on Twinflower. SNH Contract R/AC3/BAT/98/59, Final Report. Scottish Natural Heritage, Perth, UK.
- Kramer, A.T., Ison, A.J., Ashley, M.V., Howe, H.F., 2008. The paradox of forest fragmentation genetics. *Conserv. Biol.* 22, 878–885.
- Les, D.H., Reinartz, J.A., Esselman, E.J., 1991. Genetic consequences of rarity in *Aster furcatus* (Asteraceae), a threatened, self-incompatible plant. *Evolution* 45, 1641–1650.
- Loiselle, B.A., Sork, V.L., Nason, J., Graham, C., 1995. Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *Am. J. Bot.* 82, 1420–1425.
- Lowe, A.J., Cavers, S., Boshier, D., Breed, M.F., Hollingsworth, P.M., 2015. The resilience of forest fragmentation genetics—no longer a paradox—we were just looking in the wrong place. *Heredity* 115, 97–99.
- Lusby, P., Wright, J., 1996. Scottish Wild Plants: Their History, Ecology and Conservation' published by The Stationary Office Limited, Edinburgh. pp. 53–55.
- Peakall, R., Smouse, P.E., 2012. GenALEX 6.5: genetic analysis in excel. Population genetic software for teaching and research—an update. *Bioinformatics* 28, 2537–2539.
- Pickup, M., Young, A.G., 2008. Population size, self-incompatibility and genetic rescue in diploid and tetraploid races of *Rutidosia leptorrhynchoidea* (Asteraceae). *Heredity* 100, 268–274.
- Scobie, A.R., Wilcock, C.C., 2009. Limited mate availability decreases reproductive success of fragmented populations of *Linnaea borealis*, a rare, clonal, self-incompatible plant. *Ann. Bot.* 103, 835–846.
- Steven, H.M., Carlisle, A., 1959. The Native Pinewoods of Scotland. Oliver and Boyd, Edinburgh, p. 368.
- Thrall, P.H., Encinas-Viso, F., Hoebee, S., Young, A.G., 2014. Life history mediates mate limitation and population viability in self-incompatible plant species. *Ecol. Evol.* 4, 673–687.
- Vallejo-Marín, M., Dorken, M.E., Barrett, S.C.H., 2010. The ecological and evolutionary consequences of clonality for plant mating. *Annu. Rev. Ecol. Syst.* 41, 193–213.
- Vandepitte, K., Roldán-Ruiz, I., Jacquemyn, H., Honnay, O., 2010. Extremely low genotypic diversity and sexual reproduction in isolated populations of the self-incompatible lily-of-the-valley (*Convallaria majalis*) and the role of the local forest environment. *Ann. Bot.* 105, 769–776.
- Wagenius, S., Lonsdorf, E., Neuhauser, C., 2007. Patch aging and the S-Allee effect: breeding system effects on the demographic response of plants to habitat fragmentation. *Am. Nat.* 169, 383–397.
- Weir, B.S., Cockerham, C.C., 1984. Estimating *F* statistics for the analysis of population structure. *Evolution* 38, 1358–1370.
- Welch, D., 2003. A reconsideration of the native status of *Linnaea borealis* L. (Caprifoliaceae) in lowland Scotland. *Watsonia* 24, 427–432.
- Wilcock, C.C., Jennings, S.B., 1999. Partner limitation and restoration of sexual reproduction in the clonal dwarf shrub *Linnaea borealis* L. (Caprifoliaceae). *Protoplasma* 208, 76–86.
- Willi, Y., Fischer, M., 2005. Genetic rescue in interconnected populations of small and large size of the self-incompatible *Ranunculus reptans*. *Heredity* 95, 437–443.
- Wroblewska, A., 2013. The phylogeographical and population genetic approach to the investigation of the genetic diversity patterns in self-incompatible clonal and polyploid *Linnaea borealis* subsp. *borealis*. *Bot. J. Linn. Soc.* 173, 64–76.
- Young, A.G., Pickup, M., 2010. Low S-allele numbers limit mate availability, reduce seed set and skew fitness in small populations of a self-incompatible plant. *J. Appl. Ecol.* 47, 541–548.
- Young, A.G., Brown, A.H.D., Zich, F.A., 1999. Genetic structure of fragmented populations of the endangered daisy *Rutidosia leptorrhynchoidea*. *Conserv. Biol.* 13, 256–265.
- Young, A.G., Brown, A.H.D., Murray, B.G., Thrall, P.H., Miller, C., 2000. Genetic erosion, restricted mating and reduced viability in fragmented populations of the endangered grassland herb *Rutidosia leptorrhynchoidea*. In: Young, A.G., Clarke, G.M. (Eds.), *Genetics, Demography and Viability of Fragmented Populations*. Cambridge Univ. Press, Cambridge, U.K., pp. 335–359.
- Young, A.G., Broadhurst, L.M., Thrall, P.H., 2012. Non-additive effects of pollinator limitation and self-incompatibility reduce plant reproductive success and population viability. *Ann. Bot.* 109, 643–653.
- Zhang, A.-Q., Xiong, Y.-Z., Huang, H.-Q., 2014. Maintenance of self-incompatibility in peripheral populations of a circumboreal woodland subshrub. *AoB PLANTS* 6 (plu063).